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Activation of Signal Transducers and Activators of Transcription 1 and 3 by Leukemia Inhibitory Factor, Oncostatin-M, and Interferon-γ in Adipocytes*

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We have recently demonstrated that signal transducers and activators of transcription (STATs) 1, 3, 5A, 5B, and 6 are expressed in both cultured and native adipocytes. Our current studies have focused on the activation of STATs 1 and 3 by leukemia inhibitory factor (LIF), oncostatin-M (OSM), and interferon-γ (IFNγ) in 3T3-L1 adipocytes. IFNγ is shown to be a potent activator of STAT 1 as indicated by both tyrosine phosphorylation and nuclear translocation. However, LIF and OSM, which are potent inducers of STAT 3, are less potent activators of STAT 1 as measured by both tyrosine phosphorylation and nuclear translocation. Both STATs 1 and 3 were translocated to the nucleus in a time-dependent fashion following LIF treatment. In addition, IFNγ resulted in a time- and dose-dependent effect on STATs 1 and 3 nuclear translocation. Growth hormone, a potent activator of STATs 5A and 5B, had a minimal effect on STAT 1 and STAT 3 tyrosine phosphorylation. Preincubation with either insulin or growth hormone had no detectable effects on the tyrosine phosphorylation or nuclear translocation of STATs 1 and 3 induced by LIF, OSM, or IFNγ. The effects of LIF and IFNγ on STAT 1 and 3 tyrosine phosphorylation and nuclear translocation were confirmed in native rat adipocytes. In 3T3-L1 adipocytes, a low level of serine phosphorylation of STAT 3 on residue 727 was observed and was markedly enhanced by insulin, LIF, or OSM. This increase in STAT 3 Ser^{727} phosphorylation was dependent upon the activation of MAPK, since the MAPK kinase inhibitor (PD98059) reduced STAT 3 Ser^{727} phosphorylation to basal levels. The inhibition of MAPK had no effect on the ability of STATs 1 and 3 to be tyrosine-phosphorylated or translocate to the nucleus. These studies demonstrate the highly specific and quantitative activation of STATs 1 and 3 by LIF, OSM, and IFNγ in adipocytes and indicate that STAT 3 is a substrate for MAPK in adipocytes.

The signal transducer and activator of transcription (STAT)[1] family of transcription factors is composed of seven family members (STATs 1, 2, 3, 4, 5A, 5B, and 6) that, in response to stimulation of various receptors, mainly those for cytokines, are phosphorylated on tyrosine residues, which causes their translocation to the nucleus. Each STAT family member shows a distinct pattern of activation by cytokines and upon nuclear translocation can regulate the transcription of particular genes (1). The order of events for STAT activation can be briefly described as follows: 1) ligand binding of cell surface receptor; 2) receptor association with a Janus kinase family member; 3) Janus kinase tyrosine phosphorylation of STAT proteins; 4) dimerization of the STATs; 5) translocation to the nucleus; and 6) DNA binding. STATs have been shown to bind at least three different DNA consensus sequences, and this binding regulates the transcription of specific genes (1, 2). It has also been demonstrated that STATs can be activated independently of Janus kinases (1, 3) and that serine phosphorylation may also contribute to the ability of STATs to regulate transcription (4, 5).

Many recent studies suggest that the activation of particular STAT proteins may vary depending upon the cell type (1). Moreover, the tissue distribution of each STAT is unique, suggesting that the regulation of tissue-specific gene expression may be a physiological role for these proteins. This is supported by numerous reports that demonstrate that particular STATs are activated differently in response to growth factors and cytokines depending upon the cell type. Transgenic knockout experiments have revealed crucial roles for each known mammalian STAT (1), and cell-specific functions for STAT family members have been identified. To date, the activation of STAT proteins in adipocytes has not been extensively investigated.

Oncostatin-M (OSM) and leukemia inhibitory factor (LIF) are recently identified cytokines that share many structural and genetic features and are both mediators of pleiotropic biological activities (6). LIF and OSM have effects on a wide variety of cell types and are known activators of STATs 1, 3, and 5 (7–9, 11–13). The receptors for these cytokines consist of the common signaling subunit, gp130, to which other subunits are added to modify ligand specificity. In adipocytes, LIF has been shown to induce changes in lipid metabolism (14–16) and modulate the transcriptional regulation of lipoprotein lipase (15). Interferon-γ (IFNγ), which is primarily known for its roles in immunological responses, has also been shown to have potent effects on adipocyte gene expression (17) and inhibit the differentiation of cultured rodent preadipocytes (18). IFNγ has been shown to activate specific proteins in certain cell types. For example, IFNγ activates STAT 1 but not STAT 3 in some

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[1] The abbreviations used are: STAT, signal transducer and activator of transcription; oncostatin-M; LIF, leukemia inhibitory factor; IFNγ, interferon-γ; MAPK, mitogen-activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; PDGF, platelet-derived growth factor; GH, growth hormone; IL, interleukin; ERK, extracellular signal-regulated kinase; PAGE, polyacrylamide gel electrophoresis.

cell types (19, 20) and activates only STAT 5 in U937 cells (21).

In our studies, we have identified LIF, OSM, and IFNγ as inducers of STAT 1 and 3 tyrosine phosphorylation and nuclear translocation in 3T3-L1 adipocytes. Highly specific and time-dependent activation of STATs 1 and 3 by LIF and IFNγ was observed. Pretreatment of adipocytes with either growth hormone or insulin had no effect on the ability of LIF, OSM, and IFNγ to result in STAT 1 and 3 tyrosine phosphorylation or nuclear translocation. We have also demonstrated that a critical serine residue in STAT 3, serine 727, is phosphorylated to a small extent in unstimulated serum-deprived 3T3-L1 adipocytes. LIF and OSM, which are known activators of the p42/44 mitogen activated protein kinase/extracellular regulated kinase (MAPK) (22, 23), markedly enhanced STAT 3 Ser^{727} phosphorylation in 3T3-L1 adipocytes. Furthermore, the induction of STAT 3 Ser^{727} phosphorylation by LIF, OSM, and insulin was dependent upon the presence of active MAPK. In summary, these studies have examined the mechanisms of STAT 1 and 3 signal transduction by potent activators of these transcription factors in adipocytes.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM) and DNase I were purchased from Life Technologies, Inc. Bovine and fetal bovine serum were obtained from Sigma and Life Technologies, respectively. Epidermal growth factor (EGF) was purchased from Biosource International and Sigma. Murine granulocyte-macrophage colony-stimulating factor (GM-CSF) and murine LIF were from Life Technologies. Insulin, OSM, platelet-derived growth factor (PDGF-BB), and growth hormone (GH) were purchased from Sigma. Murine IFNγ, insulin-like growth factor I, interleukin (IL)-6, and IL-4 were purchased from Boehringer Mannheim. PD98059, the mitogen-activated protein kinase kinase inhibitor, was purchased from Research Biochemicals International. The non-phospho-STAT antibodies were monoclonal IgGs purchased from Transduction Laboratories or polyclonal IgGs from Quality Controlled Biochemicals Inc. Highly phosphospecific polyclonal antibodies for STAT 1 (Tyr^{701}) and STAT 3 (Tyr^{705} and Ser^{727}) were provided by Quality Controlled Biochemicals. Antiactive dual phosphospecific MAPK (ERK1/ERK2) antibody was a polyclonal IgG from Promega, and ERK 1/ERK2 was a rabbit polyclonal antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Culture—Murine 3T3-L1 preadipocytes were plated and grown to 2 days postconfluence in DMEM with 10% bovine serum. Medium was changed every 48 h. Cells were induced to differentiate by changing the medium to DMEM containing 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1.7 μM insulin. After 48 h, this medium was replaced with DMEM supplemented with 10% fetal bovine serum, and cells were maintained in this medium until utilized for experimentation. Prior to experimentation, cells were serum-deprived in DMEM containing 0.2% fatty acid-free and growth factor-depleted bovine serum albumin (Sigma) for 12 h as we have previously described (24).

Preparation of Whole Cell Extracts—Serum-deprived 3T3-L1 adipocytes were rinsed with phosphate-buffered saline and then harvested in a nondenaturing buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 1 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, and 2 mM sodium vanadate. Samples were extracted for 30 min on ice and then centrifuged at 15,000 rpm at 4 °C for 15 min. Supernatants containing whole cell extracts were analyzed for protein content using a BCA kit (Pierce) according to the manufacturer's instructions.

Preparation of Nuclear/Cytosolic Extracts—Serum-deprived 3T3-L1 adipocytes were rinsed with phosphate-buffered saline and then harvested in a nuclear homogenization buffer containing 20 mM Tris (pH 7.4), 10 mM NaCl, and 3 mM $MgCl_2$. Nonidet P-40 was added to a final concentration of 0.15%, and cells were homogenized with 16 strokes in a Dounce homogenizer. The homogenates were centrifuged at 1500 rpm for 5 min. Supernatants were saved as cytosolic extract, and the nuclear pellets were resuspended in ½ volume of nuclear homogenization buffer and were centrifuged as before. The pellet of intact nuclei was resuspended again in ½ of the original volume of nuclear homogenization buffer and centrifuged again. A small portion of the nuclei was used for trypan blue staining to examine the integrity of the nuclei. The majority of the pellet (intact nuclei) was resuspended in an extraction buffer containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 25% glycerol. Nuclei were extracted for 30 min on ice and then placed at room temperature for 10 min. Two hundred units of DNase I was added to each sample, and tubes were inverted and incubated for an additional 10 min at room temperature. Finally, the sample was subjected to centrifugation at 15,000 rpm at 4 °C for 30 min. Supernatants containing nuclear extracts were analyzed for protein content.

Rat Adipocytes—Adipocytes were isolated from the epididymal fat pads of male Sprague-Dawley rats (150–175 g) by collagenase digestion as described (25). Whole cell extracts were prepared using the nondenaturing buffer listed above. Cell fractionation was performed by homogenizing native adipocytes in nuclear homogenization buffer. Following homogenization, cytosolic and nuclear extracts were prepared from native adipocytes in an identical fashion to the procedure described above for the 3T3-L1 adipocytes.

Gel Electrophoresis and Immunoblotting—Proteins were separated in 7.5 or 12% polyacrylamide (acrylamide from National Diagnostics) gels containing SDS according to Laemmli (26) and transferred to nitrocellulose (Bio-Rad) in 25 mM Tris, 192 mM glycine, and 20% methanol. Following transfer, the membrane was blocked in 4% milk for 1 h at room temperature. Results were visualized with horseradish peroxidase-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce).

RESULTS

The phosphorylation of STATs 1 and 3 by known activators of these proteins was investigated by treating serum-deprived 3T3-L1 adipocytes with a variety of growth factors and cytokines and examining tyrosine phosphorylation and nuclear translocation. Detection of the phosphorylated forms of STATs 1 and 3 was performed with antibodies specific for the tyrosine-phosphorylated sites of these proteins on tyrosine 701 (STAT 1 Tyr^{701}) and tyrosine 705 (STAT 3 Tyr^{705}). The specificity of these antibodies was verified by first immunoprecipitating the proteins from serum-deprived 3T3-L1 adipocytes with antibodies directed to alternate sites and immunoblotting with the phosphorylation state-specific antibody (data not shown).

Whole cell, cytosolic, and nuclear extracts were analyzed from 3T3-L1 adipocytes that were treated for 15 min with IFNγ, LIF, PDGF, EGF, GH, GM-CSF, IL-6, IL-4, or insulin-like growth factor I. Immunoblotting of whole cell extracts (Fig. 1*A*) demonstrates that STAT 1 Tyr^{701} phosphorylation occurs after acute treatment with either IFNγ or LIF as indicated by blotting with the specific STAT 1 Tyr^{701} polyclonal antibody (*STAT 1 p-tyr*). The phosphorylation of STAT 3 Tyr^{705} was most potently stimulated by LIF and to a lesser extent by IFNγ treatment. However, acute exposure to PDGF, GH, IL-6, and IL-4 also resulted in detectable, albeit lower, levels of STAT 3 Tyr^{705} phosphorylation (*STAT 3 p-tyr*). Interestingly, EGF, a potent activator of STATs 1 and 3 in other cell types, had no effect on STAT 1 Tyr^{701} or STAT 3 Tyr^{705} phosphorylation in serum-deprived 3T3-L1 adipocytes. The efficacy of EGF was confirmed by examining the activation of ERK1/2 in the presence of EGF utilizing a commercially available, dual phosphospecific ERK1/2 antibody (Fig. 1*A*). Antibodies were also used to confirm the consistent protein concentrations of STATs 1 and 3 and ERK1/2 during immunoblotting (Fig. 1*A*).

In the experiment described above, cytosolic and nuclear extracts were also isolated from identically treated cells to compare the tyrosine phosphorylation and nuclear translocation of STATs 1 and 3 with the tyrosine phosphorylation we observed in whole cell extracts. As expected, both LIF and IFNγ resulted in the nuclear translocation of STAT 1 and the detection of Tyr^{701}-phosphorylated STAT 1 in nuclear extracts (Fig. 1*B*). However, not all of the activators of STAT 3 Tyr^{705} phosphorylation resulted in detectable nuclear translocation of STAT 3 (Fig. 1*B*). The *bottom two parts* of Fig. 1*B* indicate that LIF and, to a lesser extent, IFNγ resulted in the Tyr^{705} phos-

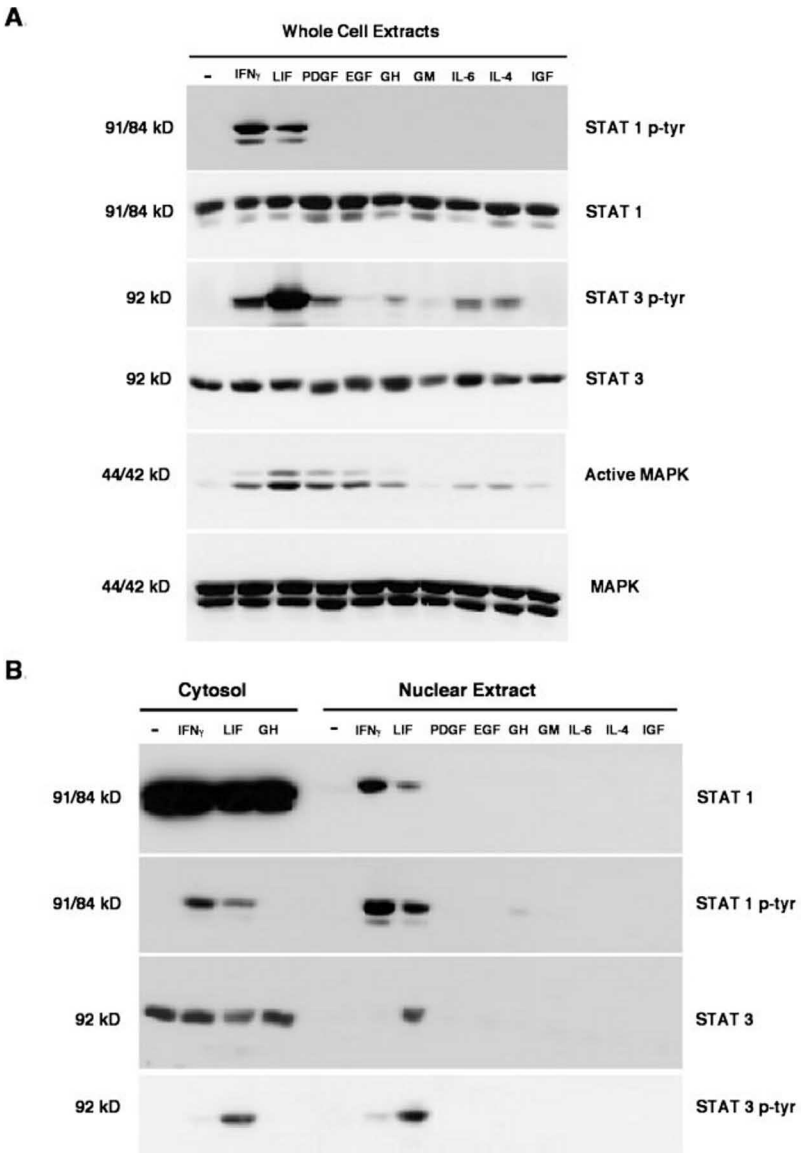

FIG. 1. **Tyrosine phosphorylation and nuclear translocation of STATs 1 and 3 in 3T3-L1 adipocytes.** Whole cell, cytosolic, and nuclear extracts were prepared from serum-deprived 3T3-L1 adipocytes following a 15-min treatment with IFNγ (100 units/ml), LIF (0.5 nM), PDGF (5 ng/ml), EGF (1 nM), GH (250 ng/ml), GM-CSF (0.5 ng/ml), IL-6 (2 ng/ml), IL-4 (5 ng/ml), or insulin-like growth factor I (15 ng/ml). Extracts were prepared as described under "Experimental Procedures." One hundred μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. The STAT 1 Tyr^{701} polyclonal (*STAT 1 p-tyr*) and STAT 3 Tyr^{705} polyclonal (*STAT 3 p-tyr*) antibodies were phosphospecific polyclonal IgGs. The STAT 1 and STAT 3 antibodies were monoclonal IgGs. The detection system was horseradish peroxidase-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce). This is a representative experiment independently performed three times.

phorylation and nuclear translocation of STAT 3. Prolonged exposures of these STAT 3 immunoblots indicated the presence of STAT 3 in the nucleus following LIF and IFNγ stimulation and to a much lesser extent by GH stimulation (data not shown). Yet, there was no detectable STAT 3 in the nucleus following stimulation by PDGF, EGF, IL-4, GM-CSF, IL-6 or insulin-like growth factor I using antibodies directed against either non-phospho-STAT 3 or STAT 3 Tyr^{705} polyclonal antibody. Therefore, we have focused our studies on the prominent activation of STATs 1 and 3 by LIF and IFNγ.

To further clarify the specific activation of STATs 1 and 3 by IFNγ, a dose- and time-dependent analysis of nuclear translocation of adipocyte-expressed STATs was performed. Serum-deprived 3T3-L1 adipocytes were treated for 15 min with eight different doses of IFNγ (0–1000 units/ml). Cells were harvested as described under "Experimental Procedures," and cytosolic and nuclear extracts were examined for the presence of STAT family members by Western blot analysis. As indicated in Fig. 2*A*, acute IFNγ treatment resulted in a dose-dependent translocation of STATs 1 and 3 to the nucleus. However, IFNγ had no effect on STATs 5A, 5B, or 6 as indicated by the absence of these proteins from the nucleus. In addition, adipocytes were treated with 100 units/ml IFNγ for various times, and the presence of STATs 1 and 3 in the nucleus was examined. As expected, the translocation of STATs 1 and 3 occurred between 3 and 15 min following IFNγ stimulation (Fig. 2*B*). The presence of these proteins in the nucleus was sustained for 90 min, with no detectable STAT proteins present in the nucleus after 120 min.

The specific activation of STATs 1 and 3 by LIF was also investigated by examining tyrosine phosphorylation and nuclear translocation. Serum-deprived 3T3-L1 adipocytes were treated with LIF for various periods and then fractionated into cytosolic and nuclear extracts. As indicated by immunoblotting extracts with STAT 1 Tyr^{701} polyclonal antibody and STAT 3 Tyr^{705} polyclonal antibody in Fig. 1, treatment with LIF resulted in the phosphorylation of both STAT 1 Tyr^{701} and STAT 3 Tyr^{705}. As demonstrated in the nuclear extract samples in the *top three panels* of Fig. 3, the STAT 1 Tyr^{701} polyclonal antibody is more sensitive than the non-phospho-STAT 1 antibody. However, analysis with either one of these antibodies demonstrates that the presence of STAT 1 in the nucleus is maximal following a 15-min LIF stimulation. Following a 5-min treatment with LIF, there was a marked detection of phosphorylated STATs 1 and 3 in the cytosol (Fig. 3). Notably, there was a concomitant loss of phosphorylated STATs 1 and 3 in the cytosol with the appearance of phosphorylated STATs 1 and 3 in the nucleus. A rapid phosphorylation of STAT 3 Tyr^{705} is

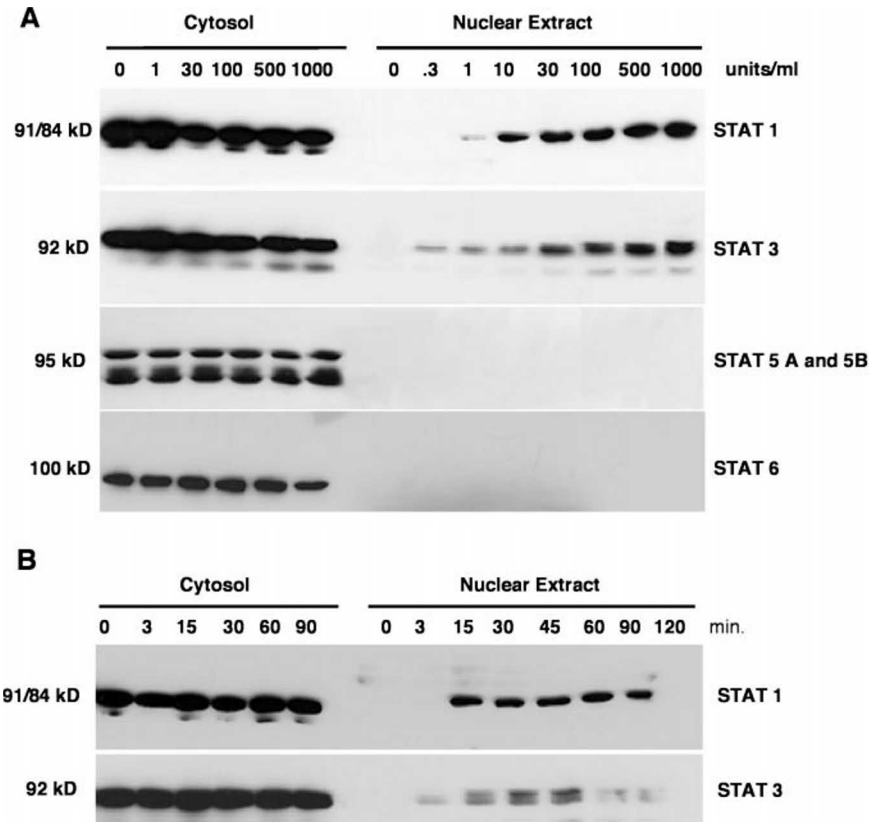

FIG. 2. **Dose- and time-dependent effect of IFNγ on STAT 1 and STAT 3 nuclear translocation.** *A*, dose dependence. Cytosolic and nuclear extracts were prepared from fully differentiated 3T3-L1 adipocytes following a 15-min treatment with various doses of IFNγ. One hundred μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. *B*, time dependence. Cytosolic and nuclear extracts were prepared from fully differentiated 3T3-L1 adipocytes following stimulation with 100 units/ml of IFNγ for various times (indicated at the *top* of *B*). One hundred μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Samples were processed, and results were visualized as described in the legend to Fig. 1. This is a representative experiment independently performed three times.

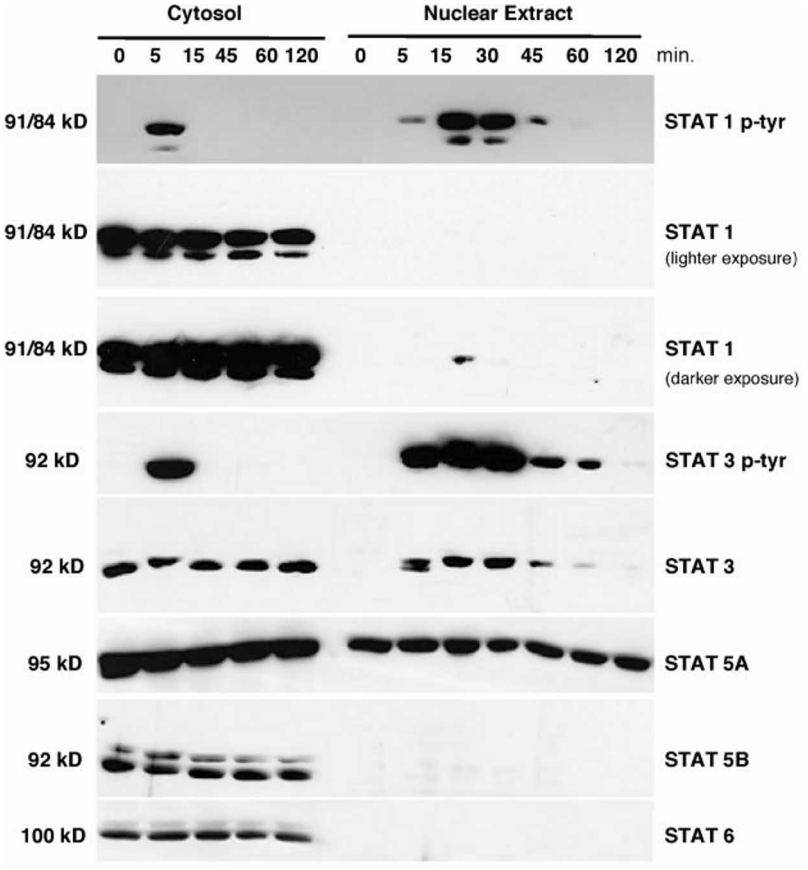

FIG. 3. **Time-dependent activation of STATs 1 and 3 by LIF in 3T3-L1 adipocytes.** Cytosolic and nuclear extracts were isolated from serum-deprived 3T3-L1 adipocytes following treatment with LIF as indicated at the *top*. One hundred μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Samples were processed, and results were visualized as described in the legend to Fig. 1. This is a representative experiment independently performed two times.

shown in the two *STAT 3 panels* of Fig. 3, with maximal accumulation in the nucleus following a 15–30-min LIF treatment. We have previously reported that STAT 5A is present in the nucleus of adipocytes under basal conditions (24), but LIF treatment does not result in changes in the amount of this protein in the nucleus (Fig. 3). Finally, neither STAT 5B nor STAT 6 protein was detectable in the nucleus following LIF treatment (Fig. 3).

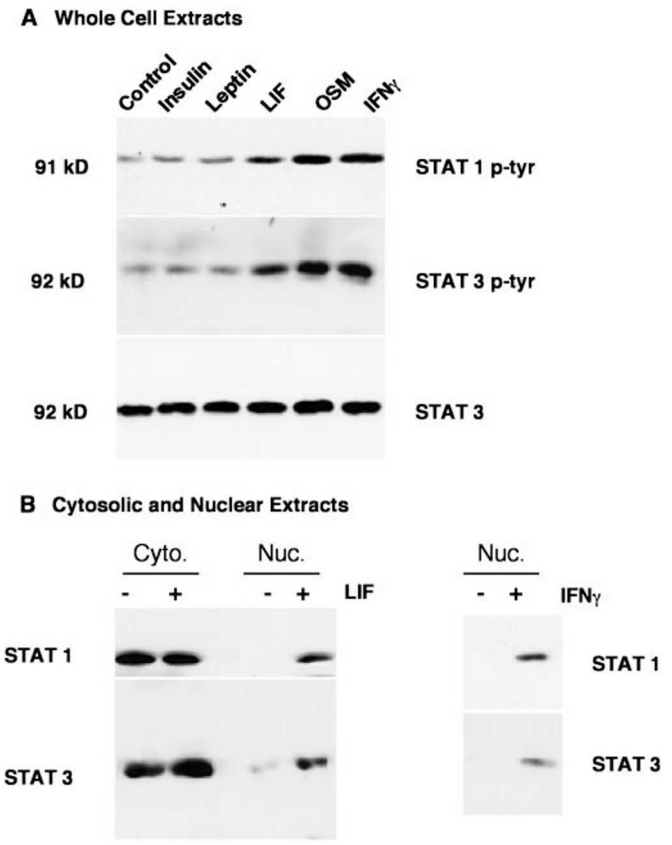

FIG. 4. **Tyrosine phosphorylation and nuclear translocation of STATs 1 and 3 by LIF and IFNγ in native rat adipocytes.** Whole cell, cytosolic, and nuclear extracts were prepared from adipocytes isolated by collagenase digestion of rat epididymal fat pads. *Panel A*, whole cell extracts were prepared following a 10-min stimulation with 50 nM insulin, leptin (25 ng/ml), OSM (0.5 ng/ml), LIF (0.5 nM), or IFNγ (100 units/ml). One hundred μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. *B*, cytosolic and nuclear extracts were prepared following a 15-min stimulation with either LIF or IFNγ. One hundred μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Samples were processed, and results were visualized as described in the legend to Fig. 1. This is a representative experiment independently performed two times.

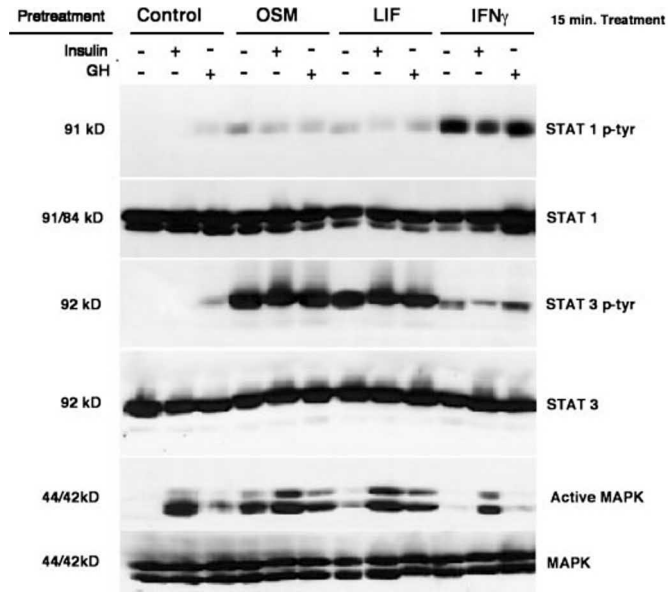

FIG. 5. **Activation of STATs 1 and 3 by OSM, LIF, and IFNγ is unaffected by preincubation with insulin or GH.** Whole cell extracts were prepared from serum-deprived 3T3-L1 adipocytes following a 20-min preincubation with either 50 nM insulin or 250 ng/ml of GH, followed by a 15-min stimulation with OSM, LIF, and IFNγ. One hundred μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Samples were processed, and results were visualized as described in the legend to Fig. 1. This is a representative experiment independently performed three times.

The activation of STATs 1 and 3 by LIF, OSM, and IFNγ was also investigated in native rat adipocytes, which were isolated by collagenase digestion of epididymal fat pads. Isolated adipocytes were stimulated for 10 min with insulin, leptin, LIF, OSM, or IFNγ. Tyrosine phosphorylation was examined by immunoblotting of whole cell extracts with the STAT 1 Tyr^{701} polyclonal and STAT 3 Tyr^{705} polyclonal antibodies. Low, but detectable, levels of STAT 1 and 3 tyrosine phosphorylation in the untreated, insulin-treated, and leptin-treated rat adipocytes were very similar. However, a substantial increase in the phosphorylation of both STAT 1 Tyr^{701} and STAT 3 Tyr^{705} by LIF, OSM, and IFNγ was observed in whole cell extracts (Fig. 4*A*). These samples were also analyzed with the STAT 3 antibody to demonstrate that the observed increases in STAT 1 and 3 tyrosine phosphorylation were not due to differences in the amount of protein present in each sample. We also examined the nuclear translocation of STATs 1 and 3 by LIF and IFNγ in native rat adipocytes. Following acute stimulation, cells were fractionated to obtain cytosolic and nuclear extracts. Acute treatment with either LIF or IFNγ resulted in a substantial increase in STATs 1 and 3 in nuclear extracts (Fig. 4*B*).

We also examined the ability of pretreatment with either insulin or GH to affect the tyrosine phosphorylation of STATs 1 and 3 by LIF, OSM, and IFNγ. Serum-deprived 3T3-L1 adipocytes were treated for 20 min with either 50 nM insulin or 100 ng/ml of GH and then treated for 15 min with OSM, LIF, or IFNγ. As previously indicated (Fig. 1), IFNγ is a potent inducer of STAT 1 Tyr^{701} phosphorylation but also results in STAT 3 Tyr^{705} phosphorylation and nuclear translocation of STAT 3 (Figs. 1 and 2). Activation of STATs 1 and 3 is clearly demonstrated in Fig. 5, where IFNγ is a potent activator of STAT 1 and LIF and OSM are potent activators of STAT 3. As indicated in Fig. 5, GH resulted in a minimal amount of STAT 1 and STAT 3 tyrosine phosphorylation, but preincubation with GH or insulin did not have any effect on the phosphorylation of STAT 1 Tyr^{701} or STAT 3 Tyr^{705} by LIF, OSM, or IFNγ. These whole extracts were also used to examine the presence of active MAPK. As indicated in Fig. 5, insulin is a potent activator of MAPK. In addition, GH, LIF, and OSM treatment also resulted in the activation of MAPK. However, neither the tyrosine phosphorylation of STATs 1 and 3 (Fig. 5) nor the presence of STATs 1 and 3 in the nucleus (data not shown) correlated with the activation of MAPK.

Recent studies have suggested that both STATs 1 and 3 are substrates of MAPK and that serine phosphorylation of STATs 1 and 3 may affect both the nuclear translocation and DNA binding of these transcription factors. Therefore, we examined the ability of OSM, LIF, and IFNγ to activate STATs 1 and 3 in the presence of the mitogen-activated protein kinase kinase inhibitor, PD98059. Serum-deprived 3T3-L1 adipocytes were pretreated with 30 μM PD98059 for 30 min and then stimulated with IFNγ alone or IFNγ and insulin for 15 min. Cytosolic, nuclear, and whole cell extracts were examined for STAT 1 and 3 tyrosine phosphorylation as shown in Fig. 6. IFNγ treatment resulted in the tyrosine phosphorylation and nuclear translocation of STATs 1 and 3, and this activation was not affected by the addition of insulin either prior to or following IFNγ treatment. In the *STAT 3 p-tyr panel*, three different treatments resulted in the presence of two distinct immunoreactive STAT 3 bands. These three treatments were from cells that were

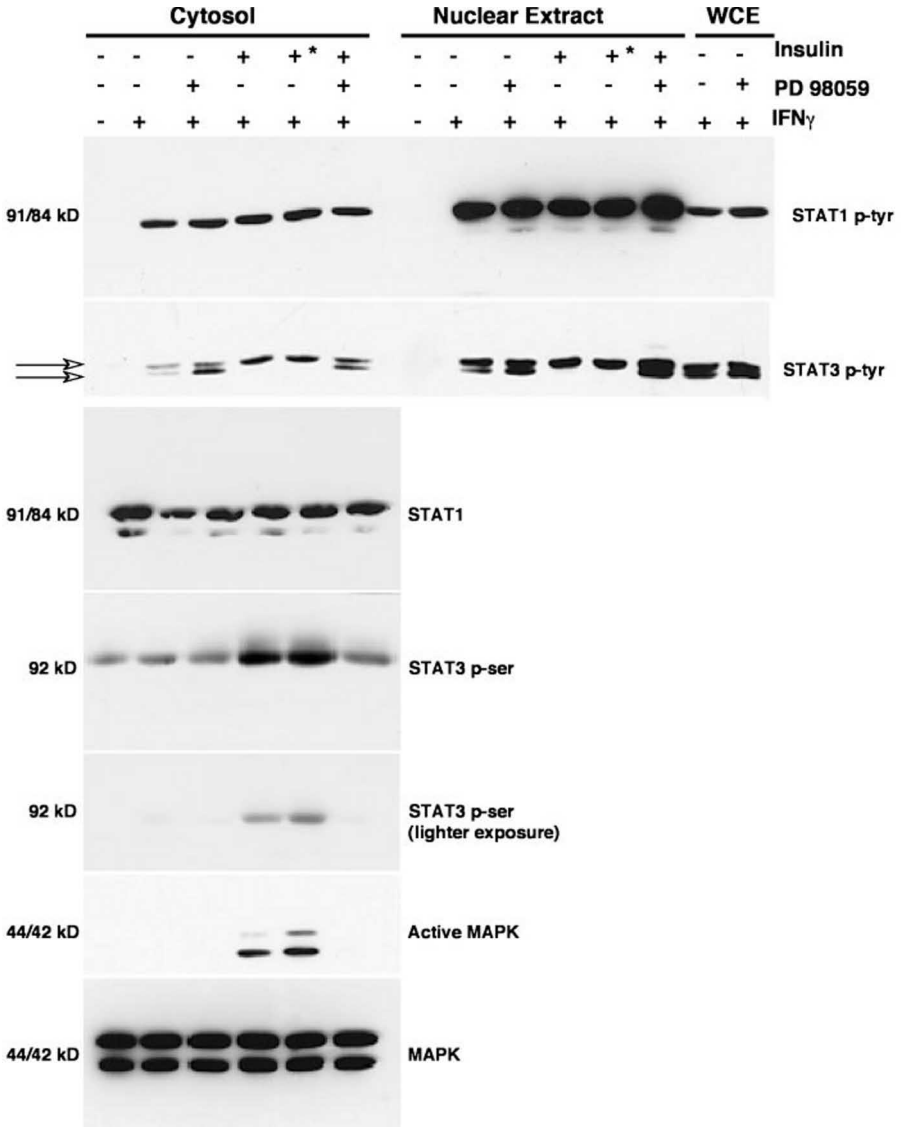

FIG. 6. **IFNγ-induced tyrosine phosphorylation and nuclear translocation of STATs 1 and 3 are independent of the activation of MAPK.** Whole cell, cytosolic, and nuclear extracts were prepared from serum-deprived 3T3-L1 adipocytes following a 15-min treatment of IFNγ (100 units/ml). Some cells were pretreated with 30 μM PD98059. Other cells were pretreated for 15 min with 50 nM insulin. For one time point, cells were treated with 50 nM insulin after the IFNγ treatment; this sample is indicated by an *asterisk* at the *top*. One hundred μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Samples were processed, and results were visualized as described in the legend to Fig. 1. This is a representative experiment independently performed three times.

stimulated with IFNγ alone, IFNγ plus PD98059, and IFNγ plus PD98059 plus insulin. The top band is STAT 3, which is both tyrosine (Tyr^{705})- and serine (Ser^{727})-phosphorylated. The lower (faster migrating) band is STAT 3, which is only phosphorylated on Tyr^{705} and is only present with IFNγ treatment. The examination of STAT 3 serine phosphorylation was performed using a phosphorylation state-specific antibody (*STAT 3 p-ser*), which only recognizes STAT 3 that is phosphorylated on serine 727. Western blot analysis revealed that STAT 3 is serine-phosphorylated to some extent in basal conditions but is markedly enhanced with the addition of insulin and the subsequent activation of MAPK (Fig. 6). This enhancement is strikingly demonstrated with a lighter exposure of the *STAT 3 p-ser* blot. The ability of insulin to increase STAT 3 serine phosphorylation is inhibited by PD98059. Also, the examination of active MAPK correlated with the enhancement of STAT 3 Ser^{727} phosphorylation and resulted in all of the STAT 3 protein migrating at the higher molecular weight band in both cytosolic and nuclear extracts. Examination of STAT 1 and MAPK in cytosolic extracts is shown to demonstrate even protein loading and the presence of unactivated proteins in untreated extracts. Insulin, a potent activator of MAPK, had no effect on STAT 1 Tyr^{701} phosphorylation or nuclear translocation. Also, the inhibition of insulin-induced MAPK activation by PD98059 had no effect on the IFNγ-induced STAT 1 Tyr^{701} phosphorylation or nuclear translocation (Fig. 6). However, treatment with insulin (either before or after acute IFNγ stimulation) resulted in all of the Tyr^{705}-phosphorylated STAT 3 migrating at the higher molecular weight. Furthermore, the ability of insulin to cause all of the STAT 3 to migrate at the higher molecular weight due to Ser^{727} phosphorylation was inhibited by PD98059 and the absence of active MAPK.

We also examined the nuclear translocation and Tyr^{701} and Tyr^{705} phosphorylation of STATs 1 and 3 after treatment with LIF and OSM in the presence of PD98059. Unlike IFNγ, LIF and OSM are potent inducers of MAPK. Serum-deprived adipocytes were pretreated with insulin (15 min) or PD98059 (30 min) and then treated with either OSM or LIF for 15 min. Pretreatment with either PD98059 or insulin had no effect on either the tyrosine phosphorylation or nuclear translocation of STATs 1 and 3 as indicated by the Western blot analysis of nuclear extracts in Fig. 7. However, acute treatment with LIF or OSM resulted in the exclusive presence of the higher migrating band of STAT 3 phosphorylated at both Tyr^{705} and Ser^{727}. In the presence of PD98059 (with or without insulin), however, there were two immunoreactive bands of STAT 3 present in nuclear extracts following acute LIF or OSM treatment (Fig. 7). An identical pattern of STAT 3 immunoreactive bands was also observed in cytosolic extracts (data not shown). Immunoblotting of nuclear extracts with the STAT 3 Ser^{727}

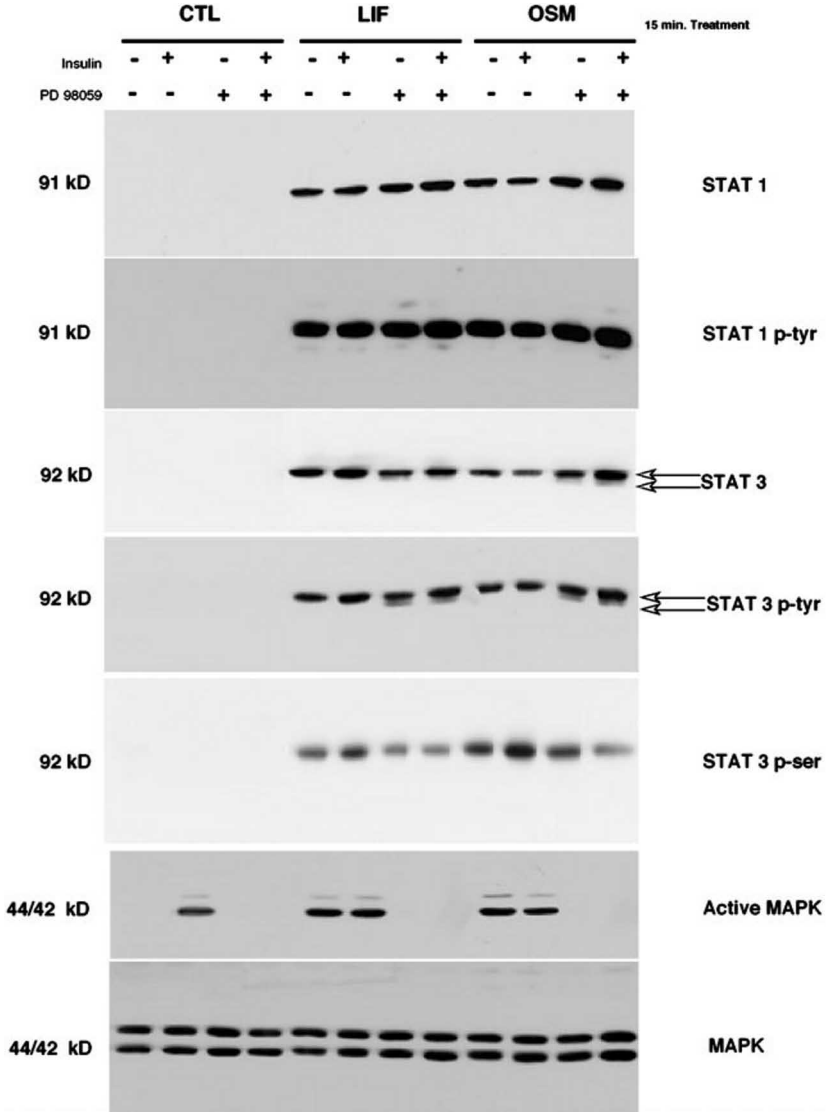

FIG. 7. **LIF- and OSM-induced tyrosine phosphorylation and nuclear translocation of STATs 1 and 3 are independent of the activation of MAPK.** Nuclear extracts were prepared from serum-deprived 3T3-L1 adipocytes following a 15-min treatment with LIF or OSM. Some cells were pretreated with 30 μM PD98059. Other cells were pretreated for 15 min with 50 nM insulin in the absence or presence of PD98059. One hundred μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Samples were processed, and results were visualized as described in the legend to Fig. 1. This is a representative experiment independently performed three times.

antibody (*STAT 3 p-ser*) revealed that there was no STAT 3 Ser^{727} in the nucleus in the absence of LIF and OSM. Although we have shown that a portion of STAT 3 is constitutively serine-phosphorylated in basal serum-deprived adipocytes (Fig. 7), this STAT 3 protein is located exclusively in the cytosol as indicated by the lack of serine-phosphorylated STAT 3 in the nucleus during unstimulated conditions (Fig. 7). The presence of Ser^{727}-phosphorylated STAT 3 is notably decreased in nuclear extracts isolated from LIF- or OSM-stimulated adipocytes that were pretreated with PD98059. As shown in Fig. 7, Western blot analysis with three different STAT 3 antibodies indicates that preincubation with either PD98059 or insulin had no effect on STAT 3 tyrosine phosphorylation and nuclear translocation. Although there is an equal amount of MAPK in each nuclear sample, active MAPK was only detected in the nucleus following insulin, LIF, or OSM treatment in the absence of PD98059 as indicated in the *bottom two panels* of Fig. 7. Although there is a much greater amount of MAPK in the cytosol, the profile of active MAPK in the nuclear extracts was identical to the pattern we observed in the cytosol (data not shown).

DISCUSSION

Of the different STAT activators we examined, IFNγ, LIF, and OSM were potent activators of tyrosine phosphorylation and nuclear translocation of both STATs 1 and 3. A detectable amount of STAT 3 Tyr^{705} phosphorylation was also observed with acute PDGF, GH, IL-4, and IL-6 treatment. However, we were unable to detect nuclear translocation under these conditions, which may be due to the sensitivity of our detection of STATs in nuclear extracts. Alternatively, weak activators of STAT 3 tyrosine phosphorylation may be insufficient to result in nuclear translocation. There is, however, no precedent for the uncoupling of STAT tyrosine phosphorylation and nuclear translocation.

We have observed distinct activation of STATs 1 and 3 in adipocytes. For example, GM-CSF has been shown to induce STATs 1 and 3 activation in polymorphonuclear leukocytes (27), but it had no effect on the activation of these STAT proteins in adipocytes. Both PDGF and EGF have been shown to activate STATs 1 and 3 in various cell types (28–31). Yet, we were unable to detect any activation of STATs 1 and 3 by EGF in 3T3-L1 adipocytes. It was observed that EGF treatment resulted in a dose- and time-dependent activation of p42/p44 (MAPK), indicating the efficacy and initiation of EGF receptor-mediated signal transduction (data not shown). Although PDGF receptor is down-regulated during 3T3-L1 differentiation, this protein is still expressed in 3T3-L1 adipocytes (32), and PDGF treatment did result in detectable levels of STAT 3 Tyr^{705} phosphorylation. It therefore seems unlikely that receptor expression alone accounts for the lack of STAT activation in adipocytes by EGF. It has recently been hypothesized that the

differential activation of STATs 1, 3, and 5 by EGF in various cell types may be attributable to the use of different members of the receptor family with different affinities for the various STAT proteins (1). These studies and our results suggest that activation of STATs is dependent upon other factors in addition to the presence of the receptor and may account, in part, for the highly specific, quantitative, and distinct activation of STATs 1 and 3 that we have observed in adipocytes.

This is the first study to demonstrate a time- and dose-dependent activation of STATs 1 and 3 by LIF and IFNγ in adipocytes. Interestingly, STAT 3 is not responsive to IFNγ in some cell types (19, 20), but IFNγ can activate STAT 5 in U937 cells (21). However, our results clearly demonstrate a time- and dose-dependent activation of both STAT 3 Tyr^{705} phosphorylation and nuclear translocation by IFNγ. In addition, IFNγ treatment did not result in the activation of either STATs 5A or 5B in adipocytes. The ability of STAT 3 to respond to IFNγ in adipocytes is probably a result of the specificity of STAT activation, which varies depending upon the cell type and may be attributed to receptor expression, binding affinities, the presence or absence of other signaling proteins, or STAT dimer composition.

Both LIF and OSM have been shown to induce STAT 5 activation in some cell types (8, 9, 11), but these growth factors did not result in STAT 5 activation in adipocytes as determined by a lack of nuclear translocation of STAT 5. However, both LIF and OSM were capable of resulting in STAT 1 Tyr^{701} and STAT 3 Tyr^{705} phosphorylation and nuclear translocation in 3T3-L1 and native rat adipocytes. We have found that LIF and OSM are potent inducers of STAT 3 tyrosine phosphorylation and nuclear translocation but also result in an activation of STAT 1. These findings are similar to those of Jenab and Morris (12), which demonstrated considerable activation of STATs 3 by LIF but significantly less activation of STAT 1 by LIF in rat Sertoli cells. Quantitative activation of the JAK-STAT pathway has also been observed in the signaling of GH, which results in 15 times more tyrosyl-phosphorylated JAK2 than either LIF or IFNγ treatment (33). Since the receptors for LIF and OSM consist of the common signaling subunit, gp130, to which other subunits are added to modify ligand specificity, we would hypothesize that the distinct activation of STATs 1 and 3 by these cytokines in adipocytes is due to unique ligand specificity.

We examined the ability of two adipogenic factors, GH and insulin, to affect the IFNγ-, LIF-, and OSM-induced Tyr^{701} and Tyr^{705} of STATs 1 and 3, respectively. GH is a potent activator of both STATs 5A and 5B in 3T3-F442 preadipocytes (34) and 3T3-L1 adipocytes (24). Insulin alone does not result in the nuclear translocation of any adipocyte expressed STAT in 3T3-L1 adipocytes (24). Although both insulin and GH resulted in MAPK activation, neither one of these agents resulted in an attenuation or enhancement of STATs 1 and 3 phosphorylation by LIF, OSM, or IFNγ. However, in rat hepatoma cells, insulin was demonstrated to antagonize the IL-6-induced activation of STAT 3 (35). We were unable to observe any ability of insulin to modulate STAT 3 Tyr^{705} phosphorylation or nuclear translocation in adipocytes. Our results indicate that activation of STATs 5A and 5B by GH does not inhibit the activation of STATs 1 and 3 and demonstrate that a number of activated STAT family members can be present in the adipocyte nucleus at one time.

Recent studies have indicated that MAPK (ERK1/ERK2) may be involved in the STAT-induced regulation of transcription and that maximal activation of STATs 1 and 3 requires both tyrosine and serine phosphorylation (4, 36–38). A requirement of serine phosphorylation of STATs 1 and 3 for the formation of STAT-promoter complexes has been demonstrated for cytokines such as LIF, OSM, IL-6, and ciliary neurotropic factor, which utilize the common signaling subunit, gp130 (36). In addition, the activation of MAPK has been shown to be required for the OSM-induced regulation of the matrix metalloproteinase gene 1 by STATs 1 and 3 (36). It has also been clearly demonstrated that STAT 3 is serine-phosphorylated by insulin in adipocytes (39). However, the role of STAT serine phosphorylation is still very controversial.

Our studies, therefore, examined the activation of MAPK by activators of STATs 1 and 3 in the presence of insulin, PD98059, and insulin plus PD98059. We were able to determine that STAT 3 was phosphorylated to a limited extent on Ser^{727} under basal conditions in adipocytes. Constitutive serine phosphorylation of STAT 1 and 3 has also been observed in B lymphocytes (40), and STATs 5A and 5B are constitutively serine-phosphorylated in mammary epithelial cells (38). We observed an insulin-dependent enhancement of STAT 3 Ser^{727} phosphorylation concomitant with an induction of MAPK activation. However, the phosphorylation of STAT 3 Ser^{727} was not required for STAT 3 to be tyrosine-phosphorylated; nor was it required for nuclear translocation. Clearly, our results suggest that STAT 3 is serine-phosphorylated by MAPK in cultured adipocytes. This is supported by a recent study that demonstrated that the ERK family of MAP kinases, but not c-Jun N-terminal kinase or p38, could specifically phosphorylate STAT 3 on serine 727 in response to growth factors (41). Yet, these data do not rule out the possibility that other kinases may also participate in the regulation of STAT serine phosphorylation. We have been unable to investigate STAT-DNA binding, since the genes that STATs regulate in adipocytes have not been identified. However, our results demonstrate that the phosphorylation of STAT 3 Ser^{727} is not required for Tyr^{705} phosphorylation or nuclear translocation. It is well documented that STATs must be tyrosine-phosphorylated and form dimers prior to translocation. Our results prove that serine phosphorylation of STAT 3 does not affect either tyrosine phosphorylation or nuclear translocation and support a recent study that demonstrated that serine phosphorylation has no influence on DNA binding of either STAT 1 or 3 (10). The issue of STAT 1 serine phosphorylation is a bit more complicated than that observed for STAT 3. To this point, the kinase responsible for STAT 1 Ser^{727} phosphorylation has not been identified. In contrast to STAT 3, STAT 1 appears to be a poor substrate for all MAPK superfamily members (41). Also, the lack of a discernible gel shift upon serine phosphorylation would necessitate that a specific STAT 1 Ser^{727} antibody be used. Work to further clarify the regulation of STAT 1 Ser^{727} phosphorylation is ongoing, but we have demonstrated that STAT 1 Tyr^{705} is independent upon the activation of MAPK.

In summary, we have examined the tyrosine phosphorylation and nuclear translocation of STATs 1 and 3 by potent activators of these transcription factors in fat cells. These studies have revealed that the activation of STATs 1 and 3 in adipocytes is highly specific and significantly different from the activation of these STATs in other cell types. These studies provide a basis for determining the function of STATs 1 and 3 in adipocytes, since there are numerous unanswered questions about the activation of STATs in adipocytes. For example, is there a physiological consequence of the quantitative activation of STATs 1 and 3 by LIF, OSM, and IFNγ? To address this and similar questions, we must identify the genes that are regulated by STATs in adipocytes and examine the protein composition of STAT DNA binding complexes and their ability to regulate transcription. We are in the process of experimentally addressing this and other hypotheses concerning the physiological activation and function of STATs in adipocytes.

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